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PRIMER NOTE

Isolation of polymorphic microsatellite markers from the malaria vector *Anopheles darlingi*

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Abstract

High molecular weight DNA was extracted from the primary Neotropical malaria vector, *Anopheles darlingi* from Capanema, Pará, Brazil, to create a small insert genomic library, and then a phagemid library. Enriched sublibraries were constructed from the phagemid library using a microsatellite oligo primed second strand synthesis protocol. The resulting 242 760 individual clones were screened. The mean clone size of the positive clones was 302 bp. Flanking primers were designed for each suitable microsatellite sequence. Eight polymorphic loci were optimized and characterized. The allele size ranges are based on 253 samples of *A. darlingi* from eastern Amazonian and central Brazil.

Keywords: *Anopheles darlingi*, malaria vector, microsatellites

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Anopheles darlingi is the primary malaria vector in the Neotropics. Considerable heterogeneity has been documented across its broad range leading to the suggestion that cryptic species may exist. A study incorporating 15 samples from Belize to southern Brazil, using isozymes, morphology, random amplified polymorphic DNAs (RAPDs) and internal transcribed spacer (ITS2) sequences, found moderate to high levels of gene flow, concluding that *A. darlingi* is a single species (Manguin *et al.* 1999). Isolation by distance has been demonstrated in *A. darlingi* using mitochondrial DNA (mtDNA) restriction fragment length polymorphism (RFLP) markers (Conn *et al.* 1999), and the ITS2 region shows a 4–5% divergence in southern vs. northern Brazil (Malafronte *et al.* 1999). Microsatellite markers would provide invaluable additional information for population-level studies of *A. darlingi*.

High molecular weight DNA was extracted from *A. darlingi* collected in Capanema, Brazil using standard phenol:chloroform:iso-amyl alcohol (PCI) and ethanol precipitation (Sambrook *et al.* 1989). A small insert genomic library was constructed by digesting genomic DNA with *Dpn*II (New England Biolabs) for 1 h at 37 °C. Digested DNA was electrophoresed on a 1.0% SeaPlaque GTG (FMC, Inc.) low melting temperature agarose gel. DNA fragments, 200–1000 bp in length, were isolated from the gel (Glenn & Glenn 1994) and ligated into the phagemid pBluescript II KS + (Stratagene Inc.) at the *Bam*HI site using T4 DNA ligase (USB Inc.). The phagemid library was electrotransformed into *Escherichia coli* strain XL1-Blue (Stratagene Inc.) using a Bio-Rad Gene Pulser I (Bio-Rad Laboratories Inc.).

Enriched (marker selected) sublibraries were then constructed from the phagemid library using a microsatellite oligo primed second strand synthesis protocol (Ostrander *et al.* 1992). The phagemid library was electrotransformed into electrocompetent ER2379 *E. coli* host (Pulido & Duyk 1994) resulting in a library of approximately 242 760 individual clones. From this, enriched sublibraries were constructed using $[TG]_{18}$, $[AC]_{18}$, and $[GA]_{18}$ primers as described in Ostrander *et al.* (1992). A small aliquot of each enriched sublibrary was electrotransformed into XL1-Blue *E. coli* (Stratagene, Inc.) for screening.

Enriched sublibraries were plated and transferred to nitrocellulose or nylon membranes (0.45 micron, 137 mm). Plasmid DNA was UV cross-linked to the membranes which were screened by hybridization with 3′ end-labelled

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Table 1 Repeat motif and primer sequences of microsatellite loci for *Anopheles darlingi*. GenBank accession numbers listed below each locus. Number of alleles based on the following samples: Aracanga (n = 38), Belém (n = 44), Granja Alves (n = 40), Lagoa dos Indios (n = 41), Mojú (n = 27), Peixoto (n = 23) and Santana (n = 40). Sequence interruptions between repeats denoted by a '+'

Locus	Repeat motif	Primer Sequence (5'–3')	Size of cloned allele	Alleles	No. of range (bp)	Allele size H_E	H_O
ADC01	$(GA)_{21}$	GCT CTA ATG ATG CTC GTA ACC GCT	218	44	154–246	0.942	0.673
AF322185		CAG GTA GTA GCA AGT GCG GTG TCG					
ADC02	$(GA)_{7+7}$	CAC ACT GGG GCA TCA TTC ATT TC	426	23	155–245	0.823	0.474
AF322186		CAG TGT CTA AGG ACG CAG TGT GA					
ADC28	$(GA)_9$	CTC GTC GTC AGC GTC GTG C	224	7	123–141	0.632	0.462
AF322187		CCG TTA CGC AGT GGA TGG GCA					
ADC29	$(GA)_{22}$	GAT CGT TGG CCG AGA ATG	271	28	160–228	0.912	0.492
AF322188		GCA TCG TGC ACC GTG ATT					
ADC107	$(AC)_{12}$	ATC GGT CCA CTC CCA GGC AC	228	22	183–277	0.785	0.527
AF322189		GCA TCG TAC GCT TCC ATC TCC TGC					
ADC110	$(GT)_{16}$	CCG AAC AAC AGC CAA CAG CTG TG	373	18	150–212	0.864	0.769
AF322190		CGT GTG TAA CGA TTG TGT CGA ACG					
ADC137	$(GT)_{11}$	TCT TAC GGG AAT GGT GCG ACG CTC	665	17	125–197	0.987	0.689
AF322191		TGG TCA ACA GCG TAT GGG TGG CTG					
ADC138	$(AC)_{14+3}$	CTT TGA GCC GGT GCT GTG CTG C	153	18	90–212	0.827	0.368
AF322192		GTC CTG GAG GCT GCG AGA ATG G					

[γP^{32}]-dATP oligonucleotide probes; $[TG]_{15}$, $[AG]_{12}$, and $[AC]_{12}$, and $[TC]_{15}$ (Sambrook *et al.* 1989). Clones exhibiting positive hybridization signal were picked and grown overnight at 37 °C in LB selective media (ampicillin and tetracycline). Plasmid DNA was isolated from clones by standard alkaline lysis procedure (Sambrook *et al.* 1989). To eliminate potential false positives, insert DNA from isolated plasmids was polymerase chain reaction (PCR) amplified using M13 universal primers flanking the *Bam*HI site. Amplifications were dot blotted directly onto nylon membranes, UV cross-linked and hybridized as above.

Plasmid DNA from 32 double positive clones was cycle sequenced with the ABI PRISM Dye Primer kit (Protocol P/N 402113 Revision B 1995) and M13 universal primers. Both strands were sequenced, checked by eye, and contig alignments constructed using SEQUENCHER 3.0 (Gene Codes Co.). The mean clone insert size was 302 bp (range 88–863 bp). Positive clones were determined to be unique by attempting pairwise alignments of all the nucleotide sequences flanking the repeat regions. Clones exhibiting repetitive elements less than six repeats were excluded.

For each suitable microsatellite sequence, flanking primers were designed with the aid of OLIGO 5.0 for the Macintosh (National Biosciences, Inc.). Primer pairs with similar melting points (T_m), %guanine-cytosine content, length (18–24 nt), and an annealing temperature near 55 °C were chosen for PCR multiplexing. Primers were tested in 15-μL PCRs containing 7.5 pmol primer, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 20 mM Tris-HCl, pH 8.4, 50 mM KCl, and 0.75 μL of 100-μL genomic template DNA from single mosquitoes extracted as in Collins *et al.* (1987). In each mosquito, genotypes were determined by PCR amplification (Lanzaro *et al.* 1995); the forward primer was labelled with FAM, HEX, or TET (Applied Biosystems, Foster City, CA). Amplifications were carried out on a PTC 100 MJR Research thermal cycler using these conditions: a 5-min 95 °C denaturation step followed by 30 cycles of 20 s at 95 °C, 30 s at 60 °C, and 20 s at 72 °C; then a final extension of 5 min at 72 °C. Samples were then sent to Ames, Iowa where the order of labelled fragments was determined. Products from three microsatellite loci were run per lane from the multiplexed PCR. Data were automatically collected and analysed using GENESCAN AND GENOTYPER software (PE-ABI).

Eight loci have been characterized to determine the degree of polymorphism (Table 1). In all localities, adult females were collected outdoors in 1997–98 between approximately 18:30–20:30, identified, and stored until use in 95% ethanol. The levels of heterozygosity detected ranged from 0.368 to 0.769 (Table 1), suggesting that these loci should be appropriate for additional population analysis.

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